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A Conventional Multiplex PCR assay for the detection of toxic gemfish species (*Ruvettus pretiosus* and *Lepidocybium flavobrunneum*): a simple method to combat health frauds.

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Abstract

The meat of *Ruvettus pretiosus* and *Lepidocybium flavobrunneum* (gemfishes) contains high amounts of indigestible wax esters that provoke gastrointestinal disorders. Although some countries banned the sale of these species, mislabelling cases have been reported in sushi catering. In this work, we developed a simple Conventional Multiplex PCR, which discriminates the two toxic gemfishes from other potentially replaced species, such as tunas, cod and sablefish. A common degenerate forward primer and three specie-specific reverse primers were designed to amplify Cytochrome oxidase subunit I (*COI*) gene regions of different lengths (479, 403 and 291 bp) of gemfishes, tunas and sablefish, respectively. A primer pair was designed to amplify a fragment (193 bp) of the *cytb* gene of cod species. Furthermore, a primer pair targeting the *16S rRNA* gene was intended as common positive control (115 bp). The method developed in this study, by producing the expected amplicon for all the DNA samples tested (reference and commercial), provide a rapid and reliable response in identifying the two toxic species and combat health frauds.

Keywords

Gempylidae, health frauds, mislabeling, multiplex PCR, mitochondrial genes

Introduction

Oilfish (*Ruvettus pretiosus*) and escolar (*Lepidocybium flavobrunneum*) belong to the Gempylidae family (gemfish) and are the only species of their respective genera[1]. Even though, according to FishBase, there are currently about 24 fish species under the family Gempylidae, only these two species may induce purgative effects, due to their high content of indigestible wax esters (approximately 20% of their wet weight)[2,3]. In fact, when these fishes are eaten, the wax esters, called Gempylotoxin, accumulate in the rectum, causing a particular type of oily diarrhea, called *keriorrhoea*[4]. For this reason, oilfish and escolar have been responsible for several food poisoning outbreaks[1,5]. Although *keriorrhoea* is not life-threatening, it can be risky for people with bowel sensitivity and pregnant women[5].

Misidentification and mislabeling can occur throughout the entire supply chain. However, on the basis of the available data, it seems that in most cases the toxic gemfish are substituted at the catering level. In particular, frauds take place in the sushi venues, when oilfish and escolar are sold as fillets or slices, therefore becoming difficult to identify on the basis of their morphological characteristics. Gemfish are often substituted to high valued species, such as tunas (*Thunnus* spp.) and cod (*Gadus* spp.) or sold as "gindara-snowfish", a term commonly used for sablefish (*Anoplopoma fimbria*) (Table 1SM), due to the fact that they have the same characteristic white meat[6]. Moreover, in this study we took into consideration also *Allothunnus* spp., *Auxis* spp. *Euthynnus* spp., and *Katsuwonus* spp. since the term "tunas" can also be used for tuna-like species belonging to these genera[7].

Due to the health problems associated with gemfish, Japan and Republic of Korea have banned it from the market, while other countries have issued health advisories[1,5]. In particular, in the European Union, "*fresh, prepared and processed fishery products belonging to the family Gempylidae, in particular R. pretiosus and L. flavobrunneum, may only be placed on the market in wrapped/packaged form and must be appropriately labelled to provide information to the consumers on preparation/cooking methods and on the risk related to the presence of substances*

with adverse gastrointestinal effects. The scientific name must accompany the common name on the label as well"[8]. Therefore, a proper identification of these species is required in order to protect consumers from commercial and health frauds.

Although DNA based methods are largely used for fish species authentication[9], to date only few protocols have been developed to identify these two toxic gemfish species. So far, Ling et al.[10] developed a DNA sequencing procedure, while Nebola[11] proposed a PCR-RFLP technique. Recently, a TaqMan® Multiplex Real-time PCR assay for the identification of *L. flavobrunneum* and *R. pretiosus* has been proposed[12].

In this work a simple Conventional Multiplex PCR assay was developed for the daily application in laboratory. This method, by permitting to distinguish the two toxic gemfish from the other species (cod, tunas and sablefish) allows to combat health frauds by reducing the long workflow of execution and high-costs often associated to the aforesaid DNA based methods.

Materials and Methods

Samples collection

Reference samples. 89 Fresh, ethanol-preserved or dried tissue samples of target species belonging to Gempylidae (*R. pretiosus* and *L. flavobrunneum*), Scombridae (*Thunnus spp., Euthynnus* spp*., Katsuwonus* spp*., Auxis* spp*. and Allothunnus* spp.*),* Gadidae (*Gadus spp.*) and *A. fimbria* were collected. Moreover, 58 other samples of non-target species belonging to Gadidae and Gempylidae and other species replaced with gemfish, such as *Xiphias gladius*, *Scomber* spp. and *Dissostichus* spp. have been considered. Reference samples were kindly provided by Research Institutes or directly collected in this study (Table 1).

Sushi samples. Species commonly used for the preparation of sushi and sashimi were also analyzed: in particular, 40 DNA sushi samples made of "white meat" fish species (10 *Sparus aurata,* 10 *Dicentrarchus labrax,*10 *Atheresthes stomias,*10 *Solea solea*), collected and molecularly identified in another ongoing study, were tested (Table 1).

DNA extraction and evaluation

The ethanol-preserved samples were re-hydrated in 100 mM TRIS-base (pH 7.8) for 30 min at room temperature. Total DNA extraction was performed from at least 20 mg of tissue following the protocol proposed by Armani et al.[13]. The amount of DNA was determined with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) by measuring the absorbance at 260 nm.

Primers projecting

Initially, 20 sequences (when available) of the mitochondrial *Cytochrome Oxidase subunit I* (*COI*) gene of all the species included in the study were randomly selected among those available in GenBank and BOLD (Table 2SM).

Parameters considered during primer design. High quality primers were designed considering three main parameters: (i) the specificity of the primers towards the target sequence, that is the ability of the primers to amplify only the intended target (and obviously not to amplify any unintended ones) by only matching to the target sequence. The selection of the conserved or polymorphic regions for designing common or genus-specific primers was carried out by aligning the sequences with Clustal W in BioEdit version 7.0.9[14]. In particular, (when possible) specific primers with at least 3 mismatches were designed; (ii) the amplicon length, which has to be different between target DNA of at least 50bp, in order to easily discriminate them on an electrophoresis gel, was also selected taking into consideration the level of DNA degradation of the samples to analyze. In fact, DNA degradation has also been observed in fresh and frozen products[15,16]; (iii) the annealing score assigned by the Multifunctional Oligo Property Analysis Tool (MOPS) (available at https://ecom.mwgdna.com/services/webgist/mops.tcl) on the basis of the primers overall characteristic (melting temperature, GC content, molecular weight, extinction coefficient and tendency to form dimers). Only the primers with a score lower than 15 were chosen.

Specific primers were produced for the amplification of target regions on the *COI* gene for gemfishes, sablefish, and tunas. On the contrary, due to the impossibility to design high quality primers on this gene, the *cytb* gene was selected as alternative target for cod species. Also in this

case, the primers were designed on the basis of the aforesaid parameters. Moreover, the possibility to choose the *cytb* gene instead of the *COI* gene for satisfying the specific requests was evaluated through a intra and inter-specific analysis of both the *COI* and *cytb* sequences of cod species and of other related Gadidae species (Table 2SM). In details, their pairwise distances was calculated with the Kimura's two parameter model[17], using the software MEGA 6.0[18].

Genera-specific and Species-specific primers targeting the COI and the cytb genes. As for the *COI* gene, one degenerated common forward primer (For-COI) and 4 reverse primers (Gem-COIR, Sable-COIR.1, Sable-COIR.2 and Tunas-COIR) were produced (Table 2). With regard to the *cytb* gene, three forward primers (Cod-cytbF, Cod-cytbF.1 and Cod-cytbF.2) and one reverse primer (Cod-cytbR) were designed (Table 2).

Control PCR Primers Targeting the 16S rRNA Gene. With the aim of amplifying a control fragment shorter than the specific bands of all the species considered, three pairs of *16Sr RNA* primers have been designed (Table 2).

Primers assessment and final multiplex PCR protocol

Primers assessment and selection. The amplification specificity of both *COI* and *cytb* primers was preliminarily evaluated before mixing them together in a multiplex reaction. In the case of the *COI*, the common forward primer (For-COI) was tested on the DNA of all the target reference species in a single reaction with each of the reverse primers designed. In the same way, the *cytb* forward and the *cytb* reverse primers were tested on all the target DNA samples of the species included in the study. The primers able to amplify the target sequences were then tested on the DNA of all the non-target species, in order to verify their specificity. Thus, the primers that did not produce unspecific bands were selected and mixed together. In the same way, the three couples of *16S rRNA* control primers were initially tested on all the reference DNA samples, in order to assess the best one. During the first multiplex reaction trial, in which the *16S rRNA* control primers have not been included, all the specific *COI* and *cytb* primers were used at the same concentration (150 nM). Subsequently, the concentrations were modified with the aim to obtain bands of similar

intensity. During this analytical step, which still did not include the *16S rRNA* control primers, the best concentration of each specific primer was set (range 75-400 nM). Therefore, the selected couple of *16S rRNA* control primers (16Sfor.3 and 16Srev.3) (Table 2) was included in the multiplex reaction after different concentration trials (from 100 to 200 nM). Finally, the concentration of both *COI*/*cytb* specific and *16s rRNA* control primers was further finely adjusted.

Final Multiplex PCR protocol. The following final Multiplex PCR protocol has been selected: 20 µL of reaction volume containing 2 µL of 10× buffer (Fisher Molecular Biology, Trevose PA, U.S.A.) with the standard concentration of $MgCl_2$ (1.5 mM), 400 µM of each dNTP (dNTPmix, Euroclone S.p.A-Life Sciences Division, Pavia, Italy), 400 nM of FORC1, 150 nM of Gem-COI, 325 nM of Sable-COI2, 125 nM of Tunas-COI, 35 nM of Cod-CytbF.2, 35 nM of Cod-CytbR, 160 nM of 16Sfor.3 and 160 nM of 16Srev.3, 25 ng/µL of BSA (Purified BSA 100× , New England BIOLABS Inc. Ipswich, MA, U.S.A.), 1 U of Perfect*Taq* DNA Polymerase (5Prime, Gaithersburg, USA), 50 ng of DNA and DNase free water (Water Mol. Bio. Grade, DNase–RNase and Protease free, 5Prime GmbH, Hamburg, Germany) with the following cycling conditions: denaturation at 94 °C for 3 min; 40 cycles at 94 °C for 20 s, 48 °C for 25 s, and 72 °C for 10 s; final extension at 72 °C for 5 min. The amplified PCR products were visualized by standard gel electrophoresis in a 4% agarose gel (GellyPhor, Euroclone).

Validation of the final multiplex PCR on sushi samples. The developed final multiplex PCR assay was tested for validation on the DNA samples of white meat species used in sushi production (species reported in white boxes in Table 1).

Amplification of the COI and cytb genes from reference DNA samples of target species.

The *COI* and *cytb* genes were amplified from 53 samples of DNA of the species extracted in this study according to the gene selected (see s*ection Parameters considered during primer design*). In particular, the primers FISH-BCL (5'-TCAACYAATCAYAAAGATATYGGCAC-3') and FISH-BCH (5'-ACTTCYGGGTGRCCRAARAATCA-3')[19], tailed with M13 by Steffens[20] were selected for the amplification of a fragment of ~700 bp of the *COI* gene of 45 DNA samples and the primers

GluFish-F (5'-AACCACCGTTGTTATTCAACTACAA-3') and THR-Fish-R (5'-ACCTCCGATCTTCGGATTACAAGACC-3')[21] were used to amplify a region of 1235 bp, including the *cytb* gene of 8 DNA samples. In the case of the *cytb* gene, the samples that failed the amplification were subsequently amplified using internal reverse primer CytBI-4R (5'-AGGAAGTATCATTCGGGCTTAATATG-3')[21] in combination with the same forward primer. The reaction solution was 20 µL in volume and contained 2 µL of 10× buffer (5Prime, Gaithersburg, USA) including 1.5 mM $MgCl_2$, 200 µM dNTPs (dNTPmix, Euroclone S.p.A-Life Sciences Division, Pavia, Italy), 300 nM each primer, 25 ng/µL BSA (Purified BSA 100×, New England BIOLABS® Inc. Ipswich, MA, USA), 1.25 U Perfect*Taq* DNA Polymerase (5Prime, Gaithersburg, USA), 100 ng DNA and DNase free water (Water Mol. Bio. Grade, DNase–RNase and Protease free, 5Prime GmbH, Hamburg, Germany), according to the following PCR cycling profile:

COI gene: denaturation at 94°C for 3 min; 45 cycles at 94°C for 30 s, 53°C for 30 s, 72°C for 35 s; final extension at 72°C for 10 min.

cytb gene: denaturation at 94°C for 3 min; 45 cycles at 94°C for 25 s, 55°C for 25 s, 72°C for 45 s; final extension at 72°C for 10 min.

Five µL of PCR products were checked by electrophoresis on a 2 % agarose gel and the presence of expected amplicons was assessed by a comparison with the standard marker SharpMass™50-DNA ladder (Euroclone, Wetherby, UK). PCR products were purified and sequenced by High-Throughput Genomics Center (Washington, USA). The obtained sequences were analyzed as reported in Armani et al.[22] and deposited on GenBank via EBI. Overall, 51 sequences have been deposited (43 of the *COI* gene and 8 of the *cytb* gene) (Table 1). The comparison of these sequences with those available on the databases, the purpose of which was to assess their reliability, was carried out by a BLAST analysis on GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and, only for *COI* gene sequences, also using the Identification System (IDS) on BOLD (Species Level Barcode Records) (http://www.boldsystems.org/index.php/IDS_OpenIdEngine). Moreover, all these

sequences were also analyzed with those retrieved from the databases and evaluated to verify the presence of polymorphisms that could affect primers' annealing specificity.

Results and Discussion

Selection of the target species

Among the DNA-based analysis methods, multiplex PCR is one of the preferred technique, due to its rapidity and simplicity of execution and to the fact that it can identify more than one species in a single reaction[23]. However, the number of species included in the assay undoubtedly influences its effectiveness. In fact, the amount of primers utilized in the reaction mix should not be too high, to avoid dimer formation and consequently negatively influence the amplification output. Therefore, considering that a selective preliminary choice of the target species represented one of the most important step to develop an efficient identification method, we planned the assay in order to only include the species with a high probability to be involved in the fraud.

Gemfish. R. pretiosus, L. flavobrunneum, T. atun, and *R. solandri* are the only commercial species in the Gempylidae family (fishbase.org), but, among them, only *R. pretiosus* and *L. flavobrunneum* are toxic[1] and frequently commercialized. For this reason, they were primarily chosen as toxic target species for the assay. Then, other species more frequently reported as replaced by toxic gemfishes were also selected (Table 1SM).

Tunas: tuna and tuna-like species. Considering that the term tunas can be used to indicate tunafish and other similar species, such *Allothunnus* spp., *Auxis* spp., *Euthynnus* spp. and *Katsuwonus* spp.[7], other than *Thunnus* spp. we included all the aforesaid genera as target.

Originally, the most common type of tuna served in Japanese sushi and sashimi was the bluefin tuna, which encompasses three distinct species: southern bluefin tuna (*Thunnus maccoyii*), pacific bluefin tuna (*Thunnus orientalis*), and northern bluefin tuna (*Thunnus thynnus*)[24]. However, after the alarming depletion of these species and the declaration of the International Union for Conservation of Nature (IUCN), which regarded bluefin stocks as endangered (http://www.iucnredlist.org/search), species such as *Thunnus alalunga* (albacore tuna), *Thunnus*

albacares (Yellowfin tuna), and *Thunnus tonggol* (Longtail tuna) have started to be widely exploited. These three species meat have a very light yellow or an almost white color, particularly in the case of *T. alalunga*, which is marketed as "white tuna" (http://www.atuna.com/index.php/tuna-info/tuna-species-guide). Therefore, they can be easily replaced with gemfishes. A recent study of Warner et al.[25] reported that 84% of the "white tuna" samples collected in the U.S. sushi restaurants were actually *L. flavobrunneum*. However, it is important to point out that tuna-sushi preparations can also include species not properly belonging to the genus *Thunnus,* but belonging to tuna-like species, such as *Allothunnus, Auxis, Euthynnus,* and *Katsuwonus*. The latter, in particular, is considered the seventh principal market tuna species after *T. alalunga*, *T. obesus*, *T. thynnus*, *T. orientalis*, *T. maccoyii* and *T. albacares* (ftp://ftp.fao.org/docrep/fao/007/y5852e/Y5852E18.pdf) and in sushi restaurants is commonly known as Katsuo (鰹).

It is interesting to note that gemfishes have often been sold instead of tunas due to their frequent bycatch in tuna fisheries[24]. In fact, it has been stated that oilfish and escolar, which were distributed almost worldwide (Fishbase.org), constituted more than 70% of the bycatch in this fishery[26]. This supports the hypothesis that part of these toxic species, often banned from the international markets, could enter into the supply chain in form of disguised products.

Cod species. According to a more general classification, the denomination "cod" refers to all the fishes in the family Gadidae[27]; on the contrary, the ASFIS list and Fishbase (http://www.fao.org/fishery/collection/asfis/en) requires a specific name for each species: atlantic cod for *G. morhua*, pacific cod for *G. macrocephalus* and Greenland cod for *G. ogac.* Thus, we decide to include in the assay only the "true cod" species belonging to the genus Gadus, which are *G. morhua*, *G. macrocephalus* and *G. ogac*. In fact, as for the tunas species (see *section Tunas: tuna and tuna-like species*) the *Gadus spp*. is very likely to be replaced, due to the color of its meat, which is very similar to that of the gemfishes[28]. In particular, on the basis of the data available (Table 1SM), it seems that in the most part of the cases the gemfishes are substituted to the high

valued atlantic cod[29,30] even though they are often also marketed with fake names, such as southern cod, canadian cod, codfish[31]or ruddercod[10]. Moreover, as reported by a recent study[16], a very high rate of mislabeling has been highlighted in seafood products sold as cod (or denomination similar to cod), which have often been replaced with other species of Gadiformes, but also with the species *Dissostichus* spp. and with sablefish.

Sablefish. We decided to include in the assay also the species *A. fimbria* (Order: Scorpaeniformes; Family: Anoplopomatidae), better known in the market as "sablefish" or improperly defined as "black cod" (www.fishbase.org). Sablefish is an economically important ground fish species inhabiting the continental shelf and slope of the North Pacific Ocean and the Bering Sea. It has long been popular in Japan, which is presently the world largest market for sablefish, and in the last decade the popularity of this species has highly grown in American restaurants, and stores (http://www.nytimes.com/2004/09/22/dining/22SABL.html?_r=0), where is best known as "smoked sable" in New York delis, "smoked black cod" in the Pacific Northwest (even though it is not related to codfish) (http://fishcooking.about.com/od/meetyourfish/p/sablefish.htm) or "gindara" at sushi venues level (http://www.thefishsociety.co.uk/shop/gindara-sushi-black-cod.html). However, most of the U.S. supply is exported, chiefly to Japan, in frozen form, where Japanese buyers sell it for sashimi[32]. Also for this species, the white color of the meat facilitate the illicit substitution with gemfishes[6] (Table 1SM). Despite the market for sablefish is primarily in Japan, China (Hong Kong), U. S. and Canada, this species has been considered as target for the assay due to the fact that it is increasingly involved in the European markets (http://www.bcseafood.ca/PDFs/fisheriesinfo/fishery-sablefish.pdf) and due to the chaos arising from the commercial term used to identify it. In fact, in a recent work[16], all the products sold under the Chinese name used for *A. fimbria* were identified as *Dissostichus* spp.

Selection of the non-target species

Related species of the Gempylidae and Gadidae families were collected. In particular, three non-toxic commercial species of the family Gempylidae (*Thyrsites atun*, *Rexea solandri* and *Rexea prometheoides*) were included in the study in order to increase the specificity of the assay; in this way, an intra-family distinction between toxic and non-toxic species was also possible. Moreover, six highly commercial species of the family Gadidae (the so called cod-related species) were included in the study: *Theragra chalcogramma* (Alaska pollock), *Melanogrammus aeglefinus* (haddock), *Microgadus proximus* (pacific tomcod), *Pollachius virens* (saithe), *Merlangius merlangus* (whiting), *Micromesistius poutassou* (blue whiting). All these species have been reported to be deliberately or unintentionally substituted with the "true cod" species, due to their similar organoleptic and morphological characteristics[16,33]. We did not include them in the assay as target species, in order to discriminate high valuable species from less valuable species. Moreover, other species (*Xiphias gladius*, *Scomber* spp. and *Dissostichus* spp.) (Table 1 and Table 1SM) seldom replaced with gemfishes were used. In particular, the species *Dissostichus* spp. (commonly known as patagonian toothfish), as well as the sablefish, has been reported as often improperly sold as cod[16]. Therefore, the inclusion of this species can further improve the assay with the possibility to identify frauds due to the misuse of the term cod.

White meat species used in sushi preparation. In addition, white meat species used in the preparation of sushi and sashimi in Asian restaurants in Italy, such as gilthead seabream (*Sparus aurata*), seabass (*Dicentrarchus labrax*), and two species of flat fish, such as *Atheresthes stomias* and *Solea solea* were also included in the study. Even though, to the best of our knowledge, no mislabeling have been reported for these latter four species, we cannot exclude the possibility that this kind of fraud may occur, due to the similarity of their meat with that of toxic gemfishes.

DNA gene markers and primer design

Among the different DNA markers used for fish species identification, mitochondrial DNA (mtDNA) has several advantages over nuclear DNA[9,34].

To date, the most commonly used mitochondrial genes are *COI, cytb* and *16S rRNA*[9]. While the *16S rRNA* gene is usually used for phylogenetic studies at mid-categorical levels, such as in families or rare genera, due to a lower inter-species variability[35], the *COI* and *cytb* genes, due to their comparable high inter-species variation and low intra-species variation, are the most widely used genetic markers for fish species identification and they have been used in a large number of studies applied to food inspection[15,16,36,37]. However, the introduction of DNA barcoding has determined a growth in the use of the *COI* gene as a genetic marker for species identification and for biodiversity analysis, with respect to the gene *cytb*[38].

Therefore, due to the fact that the *16S rRNA* gene is characterized by a low mutation rate, we decided to use it for the designing of the control primers. On the contrary, for the specific primers, we initially decided to choose the *COI* gene, due to the fact that its 700 bp polymorphic sequence has been largely used to discriminate closely related fish species[15,16,27,36]. Unfortunately, high scored primers (see *section Primers assessment and selection*) were easily obtained for gemfishes, sablefish, and tunas, but not for cod species. This might be attributed to the fact that the retrieved *COI* sequences were too short for containing enough polymorphic regions. In fact, the probability of finding polymorphic regions and their number is proportional to the length of the target sequences[39]. Therefore, for cod identification, the *cytb* gene was selected, since available sequences exceeded 1000 bp. This allowed to design high quality primers able to discriminate *Gadus spp*. from the other non-target species of the Gadidae family. Moreover, the inter-specific analysis values between the *cytb* sequences of *Gadus* spp. and the other cod-related species considered in the assay showed an higher percentage of variability (12%) respect to the *COI* gene (9%). One of the main points to take into consideration during the setting up a multiplex PCR is to design primers that both guarantee to specifically distinguish between different DNA target with an acceptable level of amplification efficiency. Obviously, the higher the number of mismatches within non-target species sequences, the better the specificity of the primer.

With regards to the mismatches position, several studies have shown that mismatches towards the 3'end of the primer affect the amplification performance much more than mismatches towards the 5'end[22,40,41,42]. Thus, we tried to design primers with mismatches near the 3'end of non-target species (Table 3SM). Moreover, all the primers were designed to amplify fragments of different length (range 115−479 bp) (see *section Specie-specific primers targeting the COI and cytb genes.*), in order to be able to distinguish the specific bands on the electrophoresis gel. Finally, considering that several studies reported high level of degradation even in case of fresh/frozen products[15,16], the longest amplicon obtainable was set at 479 bp.

All the sequences produced in this study showed a very high identity degree with those available on the database (Table 1) and no mutations were found on the annealing region of the primers. Unfortunately, no high quality sequences were obtained from the DNA sample belonging to *Allothunnus fallai* and *Thyrsites atun.* However, since the fact that these DNA sample gave the expected band when tested under the amplification condition of the Multiplex PCR assay, we have hypothesized that no many differences might exist between the available sequences and the samples we collected.

Specie-specific primers targeting the COI and cytb genes.

Considering the variability of the *COI* gene among the families considered, which makes difficult to find a common conserved region among different genera, we decided to design a degenerate primer (FOR-C1). The possibility to use a common forward primer highly decrease the probability of template-independent primer interactions during amplification, especially in the case of a multiplex PCR[23,39,43]. On the contrary, the reverse primers were designed on the polymorphic regions of the *COI* gene. In particular, the selected *COI* primers allowed to specifically amplify: a *COI* fragment gene of 479 bp from both the toxic gemfish species (*R. pretiosus* and *L. flavobrunneum*); of 403 bp from *A. fimbria*; and of 291 bp from tunas species included in the assay. As for the *cytb* gene, the selected couple of specific primers allowed to specifically amplify a fragment of 193 bp from all the three cod species (*G. microcephalus*, *G. morhua*, *G. ogac*) (Figure

1). As reported in Table 2, the selected forward *cytb* primer (Cod-cytbF.2) was obtained by the modification of Cod-cytbF.1. In particular, the primer selected had a bp different from the previous one different that allowed a greater degree of mismatch with the non-target species (Table 3SM).

Control primers targeting the 16S rRNA gene. Three primer pairs 16Sfor and 16Srev were designed relying on the high level of identity among all the Gempylidae, Gadidae, Scombridae, and other non-target species and "white meat" species (see *section 3.2.1*), after comparing all the sequences retrieved. All the control primers were designed to produce bands shorter than the specie-specific bands and obtainable even in case of extremely degraded DNA. In the end, the couple 16Sfor.3/16Srev.3 (amplicon length=115 bp) (Table 2) was the best in terms of amplificability and specificity. Even though the control amplicon was shorter than those produced by the specific primers, it was clearly distinguishable from the unspecific dimerization of other primers, thanks to the utilization of a 4% agarose gel. In fact, as already found[23,44], the choice of the most appropriate gel concentration is a fundamental parameter that can enhance the interpretation of the outcomes.

Multiplex PCR Assay: optimization and final protocol

Primers concentration and annealing temperature. During the first multiplex reaction trial (see *section Primers assessment and selection*), where all the *COI* and *cytb* specific primers were used at the same concentration (150 nM), the amplification output was very different between the *COI* and the *cytb* primers. In fact, while for the latter the band intensity was good (with a concentration higher than 70 ng/µl) estimated by the comparison with the bands produced by the standard electrophoresis ladder, for the *COI* primers it was slighter (35 ng/ µl or less) or, in some cases, not even perceivable. For this reason, we initially increased the concentration of the For-COI.1, mainly because the same forward primer was coupled with three different reverse primers (Gem-COIR, Sable-COIR.2, Tunas-COIR). Amplicons were obtained from all the samples with a For-COI concentration of 400 nM. However, the *COI* bands still resulted slighter than the *cytb* band. Therefore, the concentrations of all the reverse *COI* primers were subsequently modified with the aim to obtain bands of similar intensity (70 ng/µl for each band at least) with the *cytb* band. In this

388 way, all the specific primers produced the expected amplicon and no unspecific bands were
389 observed. However, we found that the introduction of the *16S rRNA* control primers considerably
390 upset the reaction output. In particular, if they were included in the reaction mix in a concentration
391 lower than 150 nM, the control bands did not appeared. On the contrary, a concentration higher than
392 175 nM determined the disappearance of some specific bands. Therefore, we have done several
393 trials in order to assess the appropriate final concentration. In fact, even when theoretically high
394 quality primers are produced, their relative concentration in the final mix must be adjusted to obtain
395 the expected outcomes[43]. Finally, the optimum reaction output was obtained with a concentration of
396 160 nM for the *16S rRNA* control primers combined with a substantial lowering of both the *COI*
397 and *cytb* primers (50 nM for the forward and 35 nM for the reverse). The melting temperature of
398 *COI*, *cytb* and *16S rRNA* primers was in the range of 47.5°C- 54.3°C (Table 2). Finally, an
399 annealing temperature of 48°C was selected due to the higher band intensity obtained from all the
400 DNA target samples (Figure 1). In this conditions, the bands were easily detectable on the gel and
401 with a similar intensity, with a DNA concentration of about 70 ng/µl.

402 ***Validation of the final multiplex PCR on sushi samples.*** Once tested on the market sushi
403 samples, made of the white meat species (Table 1), no amplification bands were obtained other than
404 the control bands.

405 ***Public health implication related to seafood mislabeling***

406 With the dynamic of cross cultural and inter-geographical food exchange, which is largely
407 supported by technological advances in food industry[9], the Japanese cuisine, and in particular the
408 sushi and sashimi culture, has been uniquely developed almost worldwide[45]. However, the removal
409 of morphological characteristics induced by preparation of these products, together with the
410 continuous entrance of new exotic species, represents the main challenges in seafood identification
411 by Food Business Operators (FBOs), consumers and official controllers[36].

412 Few years ago, Lowenstein et al.[24], stated that "*a piece of sushi has the potential to be an*
413 *endangered species, a fraud, or a health hazard*". This statement has been proved extremely

truthful by some studies available to date[25,46], which reported an high number of mislabeling at the catering level. In most of the cases, it consists in replacing valuable species with cheaper ones[9], but mislabeled fish can represent a health concern when the replacing is done with toxic species[47,48].

At present, with the progressive depletion of fish stocks of the most requested species, it is increasingly likely that "disguised toxic fish" may enter the food chain under false name. In fact, not only gemfishes have been mislabeled, but also other toxic species such the spanish mackerel labeled as kingfish or puffer fish sold as squid or cod[16,36].

Even though health frauds are less frequent than commercial frauds, due to the health implications for the consumers that often required hospitalization, gemfishes are good candidates for voluntary frauds. In fact, the Gempylotoxin may determine consequences that are mild in most cases, with gastrointestinal symptoms that commonly arise only in people with bowel sensitivity[5]. Furthermore, the symptoms are seldom long lasting and absolutely hardly deadly, contrarily to the puffer fish, whose heat stable tetrodotoxin (TTX) is potentially deadly with a minimum lethal dose, which in all adult human is estimated to be only 2-3 mg[49]. This aspect leads us to conjecture that the number of mislabeling cases currently reported could be underestimated, even due to the fact that, in case of sushi products, the portion of fish is very small and it hardly makes consumers sick.

For this reason, while all the fish belonging to the family Tetraodontidae must not be placed on the EU market[50], for fishes belonging to Gempylidae family this prohibition does not exists, yet, although health advisories have been issued[8]. For the aforesaid reasons, the availability of a rapid method for the authentication of fish products represents a pivotal aspect for the accurate labeling of seafood species and for the protection of consumers' health.

In the light of a growing globalization of seafood trade and of an increased call for safety and quality requirements, margins for mislabeling and health frauds for substitution can be significantly reduced by the use of DNA based analytical techniques, able to properly identify fish species and thus support traceability verification. This is the case of the simple Conventional Multiplex PCR developed in this study, which can be used even in routinely applications to identify not only the

toxic gemfish species, but also the other most common replaced species, such as cod, tunas and sablefish. This method, which may be considered a valuable aid to combat health frauds, may be also useful to better outline and understand the mechanisms related to the chaos affecting the commercialization of cod products.

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Yazawa of Department of Marine Biosciences, Tokyo University of Marine Science and Technology (Tokyo, Japan).

Supporting information description

Table 1SM. Studies reporting cases of fish species substitution with Gemfish.

Table 2SM. Species included in this study and corresponding COI and cytb sequences retrieved from GenBank and BOLD.

Table 3SM. Alignment between the COI and cytb primers designed in this study and the available COI and cytb gene sequences of the species included in this work. Mismatches are highlighted in grey. NAS: Not Available Sequences.

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Figure captions

Figure 1. Electrophoresis on agarose gel (4%) of the species selected as target after the Multiplex PCR; the ''genus control'' band of 115 bp is clearly visible together with the species-specific band. Line 1, *Lepidocybium flavobrunneum* (479 bp), bp); line 2, *Anoplopoma fimbria* (403 bp); line 3, *Thunnus albacares* (291 bp); line 4, *Gadus morhua* (193 bp); line 5, H2O; lines 1,2,3,4: 16S rRNA positive control bands (115 bp). DNA ladder band size: 1000 bp, 900 bp, 800 bp, 700 bp, 600 bp, 500 bp, 400 bp, 300 bp, 250 bp, 200 bp, 150 bp, 100 bp, 50 bp.

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Tables

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Order	Family	Genus/species	Research Institute	Number of specimens	FAO Area	Target gene	Sequences	GenBank sequence code	BLAST	
									GenBank	BOLD
Gadiformes	Gadidae	*Gadus macrocephalus*	Alaska Fisheries Science Center - NOAA/NMFS (Seattle WA, USA)	10	67	*cytb*	3	LN908942	100% *G. macrocephalus*	-
								LN908943	100% *G. macrocephalus*	
								LN908941	100% *G. macrocephalus*	
		Gadus morhua	Fishlab	3	27	*cytb*	3	LN908938	100% *G. morhua*	-
								LN908939	100% *G. morhua*	
								LN908940	100% *G. morhua*	
		Gadus ogac	Fisheries and Oceans, Maurice Lamontagne Institute (Mont-Joli Qc, Canada)	5	21	*cytb*	2	LN908944	100% *G.ogac*	-
								LN908945	100% *G.ogac*	
			Departamento de Bioquímica de Alimentos, Instituto de Investigaciones Marinas CSIC (Vigo, Spain)	1	21		-			
		Melanogr	Fishlab	5	21	*cytb*				

		ammus aeglefinus								
		Merlangius merlangus	Fishlab	5	21	*cytb*				
		Microgadus proximus	Alaska Fisheries Science Center - NOAA/NMFS (Seattle WA, USA)	5	67	*cytb*				
		Micromesistius poutassou	Fishlab	5	21	*cytb*				
		Pollachius virens	Fishlab	5	21	*cytb*				
		Theragra chalcogramma	NOAA Fisheries - Alaska Fisheries Science Center (Seattle WA, USA)	6	67	*cytb*				
			Alaska Fisheries Science Center - NOAA/NMFS (Seattle WA, USA)	5	67					
Perciformes	Gempylidae	*Lepidocybium flavobrunneum*	NOAA Fisheries, Pacific Islands Fisheries Science Center (Honolulu H,I USA)	2	77	*COI*	2	LN907515	100% *L.flavobrunneum*	100% *L.flavobrunneum*
								LN907516	100% *L.flavobrunneum*	100% *L.flavobrunneum*
			Australian museum	2	57		2	LN907517	100% *L.flavobrunneum*	100% *L.flavobrunneum*
								LN907518	100% *L.flavobrunneum*	100% *L.flavobrunneum*
			Biodiversity Research Center, Academia Sinica (Taipei, Taiwan)	2	-		2	LN907519	100% *L.flavobrunneum*	100% *L.flavobrunneum*
								LN907520	100% *L.flavobrunneum*	100% *L.flavobrunneum*

		Rexea prometheoides	Biodiversity Research Center, Academia Sinica (Taipei, Taiwan)	1	-	*COI*	1	LN908934	100% *Rexea* spp.	100% *R. prometheoides*
		Rexea solandri	School of Biomedical Sciences, University of Queensland (St Lucia QLD, Australia)	5	57	*COI*	5	LN907526	100% *R.solandri*	100% *R.solandri*
								LN907527	100% *R.solandri*	100% *R.solandri*
								LN907528	100% *R.solandri*	100% *R.solandri*
								LN907529	100% *R.solandri*	100% *R.solandri*
								LN907530	100% *R.solandri*	100% *R.solandri*
		Ruvettus pretiosus	Australian Museum	1	57	*COI*	1	LN907521	100% *R.pretiosus*	100% *R.pretiosus*
			Biodiversity Research Center, Academia Sinica (Taipei, Taiwan) Local Health Authority of Milan (Milan, Italy)	4 2	- 37		2 2	LN907522	100% *R.pretiosus*	100% *R.pretiosus*
								LN907523	100% *R.pretiosus*	100% *R.pretiosus*
								LN907524	100% *R.pretiosus*	100% *R.pretiosus*
								LN907525	100% *R.pretiosus*	100% *R.pretiosus*
		Thyrsites atun	Department of Chemistry, Tshwane University of Technology (Pretoria, South Africa)	1	81	*COI*	-			
	Moronidae	*Dicentrarchus labrax*	FishLab	10	34	*COI*				
	Notothenidae	*Dissostichus eleginoides*	FishLab	5	21	*COI*				
	Scombridae	*Allothunnus fallai*	Marine Vertebrate Collection Scripps	1	-	*COI*	-			

			Institution of Oceanography, University of California (La Jolla CA, USA)							
		Auxis thazard	Department of Marine Biosciences, Tokyo University of Marine Science and Technology (Tokyo, Japan)	2	61		1	LN908925	100% *A. thazard*	100% *A. thazard*
		Auxis rochei	Department of Marine Biosciences, Tokyo University of Marine Science and Technology (Tokyo, Japan)	2	61		1	LN908925	100% *A. rochei*	100% *A. rochei*
		Euthynnus affinis	Department of Marine Biosciences, Tokyo University of Marine Science and Technology (Tokyo, Japan)	1	61		1	LN908927	99% *E.affinis*	99.9% *E.affinis*
			Museum of Natural Science, Louisiana State	12	61		1	LN908926	100% *E.affinis*	100% *E.affinis*

			University (Baton Rouge LA, USA)							
		Euthynnus lineatus	NOAA Fisheries, Southwest Fisheries Science Center (La Jolla CA, USA	1	77		1	LN908928	99% *E.lineatus*	99.7% *E.lineatus*
		Katsuwonus pelamis	Joint Faculty of Veterinary Medicine Yamaguchi University (Yoshida, Japan)	3	61		2	LN908929	99% *K. pelamis*	99.8% *K. pelamis*
								LN908930	100% *K. pelamis*	100% *K. pelamis*
		Thunnus alalunga	Departamento de Geoquimica, Universidade Federal Fluminense (Niterói, Brasil)	1	41		1	LN908908	100% *T.alalunga*	100% *T.alalunga*
			This study	1	27		1	LN908909	100% *T.alalunga*	100% *T.alalunga*
		Thunnus albacares	Departamento de Geoquimica, Universidade Federal Fluminense (Niterói, Brasil)	1	41		1	LN908910	100% *T.albacares*	100% *T.albacares*
			Fishlab	1	27		1	LN908911	100% *T.albacares*	100% *T.albacares*
		Thunnus maccoyii	Institute of Metabolic Physiology, Heinrich-	1	31		1	LN908912	100% *T. maccoyii*	100% *T. maccoyii*

			Heine-Universitaet (Duesseldorf, Germany)							
		Thunnus obesus	Joint Faculty of Veterinary Medicine Yamaguchi University (Yoshida, Japan)	3	61		2	LN908913	100% *T. obesus*	100% *T. obesus*
								LN908914	99% *T.obesus*	99.8% *T.obesus*
			Departamento de Geoquimica, Universidade Federal Fluminense (Niterói, Brasil)	2	41		2	LN908915	100% *T. obesus*	100% *T. obesus*
								LN908916	100% *T. obesus*	100% *T. obesus*
		Thunnus orientalis	Department of Marine Biosciences, Tokyo University of Marine Science and Technology (Tokyo, Japan)	2	61		1	LN908917	100% *T. orientalis*	100% *T. orientalis*
			Joint Faculty of Veterinary Medicine Yamaguchi University (Yoshida, Japan)	3	61		2	LN908918	100% *T. orientalis*	100% *T. orientalis*
								LN908919	100% *T. orientalis*	100% *T. orientalis*
		Thunnus thynnus	Federation of Maltese Aquacult	5	37		2	LN908920	100% *T. thynnus*	100% *T. thynnus*
								LN908921	100% *T. thynnus*	100% *T. thynnus*

			ure Producers (Valletta, Malta)							
		Thunnus tonggol	Department of Marine Biosciences, Tokyo University of Marine Science and Technology (Tokyo, Japan)	10	61		3	LN908922	100% *T. tonggol*	100% *T. tonggol*
								LN908923	100% *T. tonggol*	100% *T. tonggol*
								LN908924	100% *T. tonggol*	100% *T .tonggol*
		Scomber scombrus	FishLab	5	27					
	Sparidae	*Sparus aurata*	FishLab	10	27					
	Xiphiidae	*Xiphias gladius*	FishLab	5	21					
Pleuronectiformes	Pleuronectidae	*Atheresthes stomias*	FishLab	10	71					
	Soleidae	*Solea solea*	FishLab	10	27					
Scorpaeniformes	Anoplopomatidae	*Anoplopoma fimbria*	University of Washington Fish Collection (Seattle WA, USA)	5	77	*COI*	3	LN908931	100% *A. fimbria*	100% *A. fimbria*
								LN908932	100% *A. fimbria*	100% *A. fimbria*
								LN908933	100% *A. fimbria*	100% *A. fimbria*

646

647 **Table 1**. Tissue samples collected in this study; dark gray boxes: target species; light gray boxes: non-target
648 species; white boxes: species used in the preparation of sushi.

649

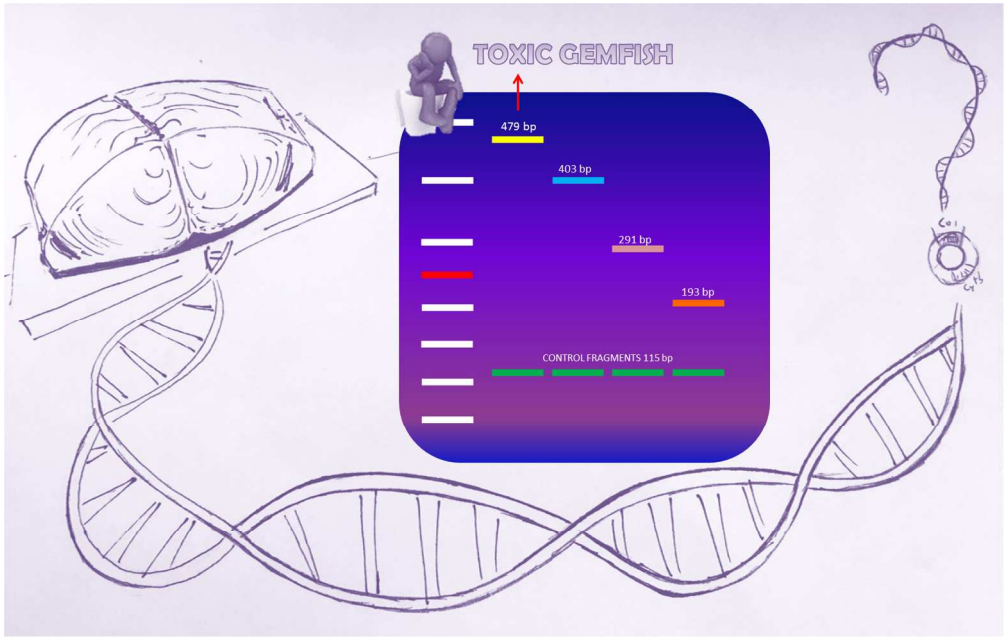
650

Primer	Sequence (5'-3')	Target species	Lenght (bp)	Position	AL (bp)	MT (°C)
For-COI	TATCTHGTATTYGGTGC	All the target species	17	5	-	47.5
Gem-COIR	TGGGAGATGGCTGCG	*R. pretiosus; L. flavobrunneum*	15	483	**479**	53.3
Sable-COIR.1	CAAGAGGGCGCCAG	*A.fimbria*	14	144	139	48.0
Sable-COIR.2	CAAGTTACTGGCGAG		15	408	**403**	47.8
Tunas-COIR	AGAAGCTAGGAGHAGAA	*Thunnus* spp.*; Allothunnus* spp*; Auxis* spp.*; Euthynnus* spp.*; Katsuwonus* spp.	17	294	**291**	48.7
Cod-cytbF	CTAGGTGGCGTACTTG	*Gadus* spp.	16	861/862	**193**	51.7
Cod-cytbF.1	CTAGGTGGCGTGCTTG		16			54.3
Cod-cytbF.2	TAGGTGGCGTGCTTG		15			50.6
Cod-cytbR	GTCCGATGATAATGAAGG		18	1054		51.4
16Sfor.1	TTRACCGTGCGAAGG	All the species included in the study	15	1010	60	49.2
16Srev.1	TGCCWTTCATACMGGTC		17	1069		51.6
16Sfor.2	GTACCTTTTGCATCATGA		18	210	90	49.1
16Srev.2	TGTCTTGGAGTAGCTC		16	299		49.2
16Sfor.3	TACGACCTCGATGTTG		16	1437	**115**	49.2
16Srev.3	TGACCTGGATTACTCC		16	1551		49.2

651

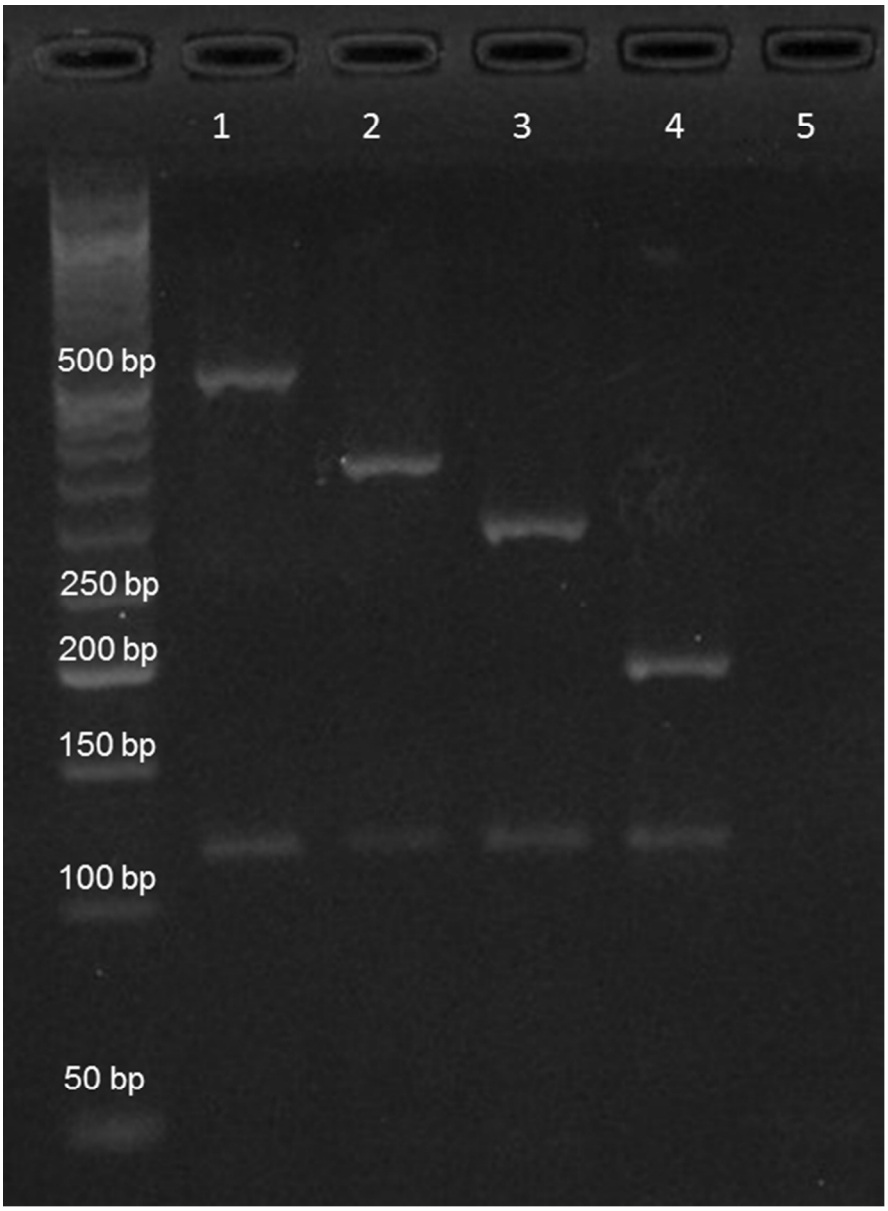
652 **Table 2.** Primer designed for the multiplex PCR. Highlighted in grey: primers finally selected. bp: base pair;
653 AL: Amplicon length; MT: Melting Temperature.

654



TOC graphic

150x95mm (300 x 300 DPI)



Electrophoresis on agarose gel (4%) of the species selected as target after the Multiplex PCR; the ''genus control'' band of 115 bp is clearly visible together with the species-specific band. Line 1, Lepidocybium flavobrunneum (479 bp), bp); line 2, Anoplopoma fimbria (403 bp); line 3, Thunnus albacares (291 bp); line 4, Gadus morhua (193 bp); line 5, H2O; lines 1,2,3,4: 16S rRNA positive control bands (115 bp). DNA ladder band size: 1000 bp, 900 bp, 800 bp, 700 bp, 600 bp, 500 bp, 400 bp, 300 bp, 250 bp, 200 bp, 150 bp, 100 bp, 50 bp.
46x63mm (300 x 300 DPI)